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Neuropathogenic murine leukemia virus TR1.3 induces selective syncytia formation of brain capillary endothelium

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Abstract

Exposure of newborn BALB/c mice to murine leukemia virus (MLV) TR1.3 induces fusion of brain capillary endothelial cells (BCEC), loss of cerebral vessel integrity, hemorrhagic stroke, and death. Although TR1.3 infects endothelial cells in multiple organs, syncytia are only observed in BCEC. To determine if viral and cellular factors are responsible for selective syncytia formation, capillary endothelial cells (CEC) from multiple organs were assayed in vitro for MLV infection and cell fusion. Following incubation with virus, all CEC were infected to an equal extent as determined by expression of MLV envelope and infectious virus production; however, MLV-induced syncytia were only observed in TR1.3-infected BCEC cultures. These in vitro results mirror the in vivo pattern of TR1.3 MLV infection and neuropathology, and definitively show that selective fusion and pathology of BCEC by MLV is determined by properties unique to BCEC as contrasted to other endothelial cell types.

Keywords: Murine leukemia virus; Retrovirus; Brain endothelium; Neuropathology; Syncytia; Cell fusion

Introduction

Endothelial cell damage can lead to the development of numerous human vascular disorders including atherosclerosis, thrombosis, hemorrhage, and sarcoma formation. In several instances, these changes are linked to, or associated with, virus infection (Benditt et al., 1983; Fabricant, 1985; Fish et al., 1998; Lathey et al., 1990; Ross, 1986). For example, infection of endothelial cells by Human Herpes Virus 8 is associated with cellular transformation, spindle cell formation, and the development of Kaposi's sarcoma (Boshoff et al., 1995; Chang et al., 1994; Moses et al., 1999), and infection by human immunodeficiency virus type-1 (HIV-1) is associated with damage to cerebral vessels and an increase in the incidence of stroke (Engstrom et al., 1989; Park et al., 1990; Qureshi et al., 1997). In most instances, the precise mechanisms responsible for virus-induced changes in endothelial cell viability and function are unknown.

Infection of capillary endothelial cells (CEC) is a prerequisite of neurological disease in several strains of murine leukemia virus (MLV), and these viruses have accordingly been used as modes of human retroviral infections (Czub et al., 1994; Hoffman et al., 1992; Lynch et al., 1991; Pitts et al., 1987; Stoica et al., 1993). The induction of neurological disease by MLV is linked to the capacity of brain capillary endothelial cells (BCEC) to support virus replication; disease results from either the direct perturbation of BCEC function (Masuda et al., 1993; Park et al., 1993) or the subsequent infection of other cell types within the CNS (Czub et al., 1994; Masuda et al., 1993; Stoica et al., 1993). TR1.3 is an acutely neuropathogenic form of Friend MLV that causes progressive, hemorrhagic stroke in BALB/c mice (Park et al., 1993, 1994a, 1994b). Exposure of neonatal mice to TR1.3 results in infection of CEC in multiple

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organs within 24–48 h; however, cytopathology is only detected within BCEC. Infected BCEC form multinucleated giant cells, termed syncytia, beginning at 4–7 days of infection. Syncytia formation both disrupts the integrity of the blood–brain barrier and initiates thrombogenic events within brain capillary vessels that lead to the death of animals at 12–18 days.

The molecular basis of TR1.3-induced syncytia formation and neurological disease lies in a single tryptophan to glycine amino acid substitution at residue 102 (W102G) within the virus envelope (Env) protein. TR1.3 is closely related by sequence to FB29 MLV, which infects but does not cause syncytia formation in BCEC (Park et al., 1994a, 1994b). Introduction of the W102G substitution in the FB29 Env protein is sufficient to reproduce BCEC fusion, cerebral hemorrhage, and death of BALB/c mice in a manner indistinguishable from that seen following TR1.3 infection. We recently demonstrated that W102G Env is inherently more fusigenic than FB29 Env in in vitro cell fusion assays (Chung et al., 1999). However, because TR1.3 infects CEC of multiple organs and yet syncytia are seen only in BCEC (Park et al., 1993, 1994a, 1994b), cellular factors may also regulate TR1.3-dependent syncytia formation.

The present studies were initiated to determine whether BCEC uniquely regulate susceptibility to syncytia formation and neurological disease. The experimental approach was to develop an in vitro model of primary mouse CEC cultures for detailed, comparative analysis of the consequences of MLV infection. Our results demonstrate that FB29 and TR1.3 established productive infection and equal levels of Env expression in all CEC cultures regardless of organ type. As seen in vivo, FB29 infection did not result in syncytia formation or other cytopathic effects in any CEC type, although TR1.3 infection induced syncytia formation and cell death selectively in BCEC. These results demonstrate that TR1.3-dependent syncytia formation is a property unique to BCEC, and establish a working model to dissect how different CEC types regulate retrovirus-dependent cytopathology.

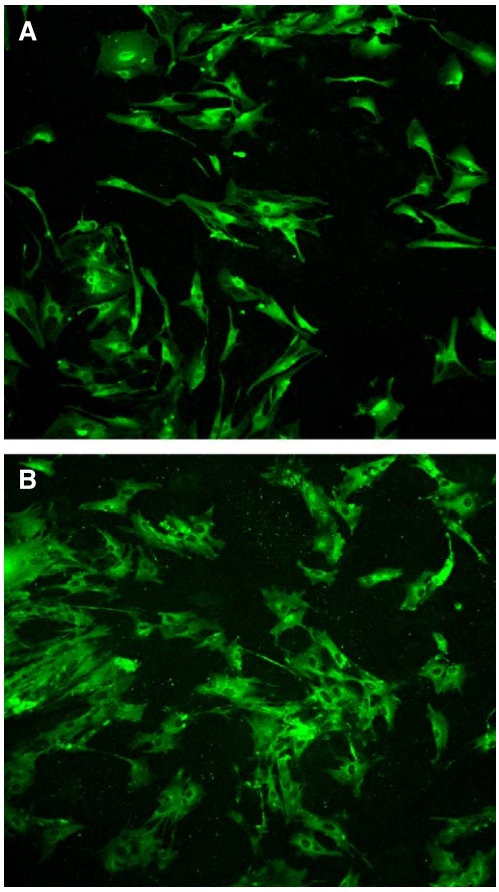

Fig. 1. Immunofluorescence detection of Factor VIII expression in primary murine BCEC and KCEC cultures. Primary BCEC (A) and KCEC (B) cultures were fixed in paraformaldehyde and then incubated with rabbit antihuman Factor VIII (1:50 dilution). After washing, antibody binding was detected (magnification, 50×) by incubation with FITC-conjugated donkey anti-rabbit IgG.

Results

FB29 and TR1.3 MLV productively infect BALB/c mouse primary CEC

As a first step in the analysis of TR1.3 cytopathology for BCEC, we investigated the ability of TR1.3 and FB29 MLV to infect primary capillary endothelial cell cultures in vitro. Primary cultures of murine CEC were established from brain, kidney, liver, and lung tissues, and cell purity was determined by immunohistochemical detection of Factor VIII expression (Hoffman et al., 1992). As shown in Fig. 1 for BCEC and KCEC, the expression of Factor VIII exceeded 95% in CEC primary cultures, and was uniform in cultures maintained for up to seven passages in vitro. Similar results were obtained in the analysis of CEC isolated from liver and lung tissues (data not shown).

In vitro proliferation of CEC was next determined by assay of ^{3}H-thymidine incorporation. Although there were subtle early differences in the proliferation of individual CEC types, all CEC examined displayed the same extent of proliferation by 48 h of culture. For example, BCEC displayed a slightly greater proliferative capacity during the first 24 h after passage than KCEC (Fig. 2); however, proliferative capacity from this point onward (up to seven passages) was not significantly different between these lines. In view of this, CEC lines were exposed to MLV at no earlier than 48 h after cell passage.

MLV infection of primary CEC was evaluated by several criteria including the expression of virus Env glycoprotein, the production of infectious virus, and the detection of provirus. Subconfluent CEC cultures were incubated with either FB29 or TR1.3 MLV at a multiplic-

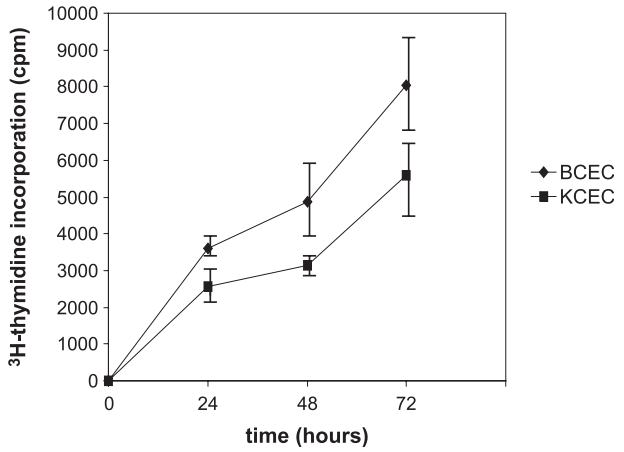

Fig. 2. Comparison of cellular proliferation in primary BCEC and KCEC cultures. [^{3}H]-thymidine incorporation of BCEC (diamond symbol) and KCEC (square symbol) was analyzed at 24, 48, and 72 h following a 6-h pulse with radiolabel. Each datum point represents the mean ± standard error of the mean (n = 6).

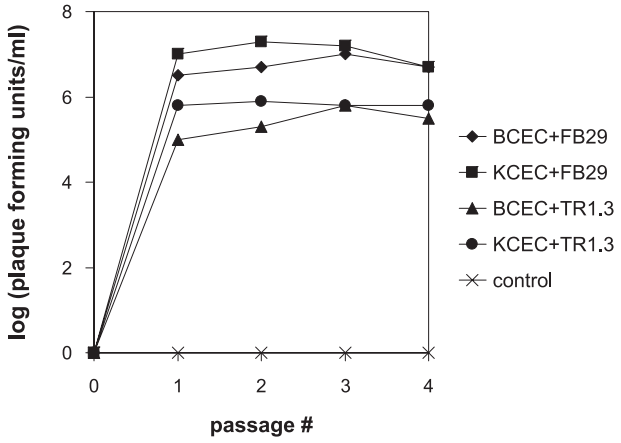

Fig. 4. Production of infectious MLV by BCEC and KCEC in vitro. Subconfluent BCEC and KCEC cultures were incubated with either TR1.3 or FB29 MLV (MOI = 1.0) and serially passed in vitro. Supernatants were collected at each passage and titered for virus production by XC-plaque assay. The data shown are representative of three independent experiments.

ity of infection of 1.0 and analyzed after one to four passages in vitro. Expression of virus Env glycoprotein gp70 was first determined by immunohistochemistry and flow microfluorimetry. Results shown in Fig. 3 demonstrate that FB29 and TR1.3 induced an equal level of gp70 expression in BCEC, KCEC, LiCEC, and LuCEC at 48 h of infection. Similar results were obtained in longitudinal analysis of infected CEC at multiple time points over four passages.

One-time infection growth curves were next generated from BCEC and KCEC cultures incubated with either TR1.3 or FB29 to verify that these cells were able to support productive virus infection. BCEC and KCEC produced indistinguishable levels of infectious virus throughout extended passage in vitro as determined by the XC plaque assay (Fig. 4). Interestingly, both BCEC and KCEC produced slightly more virus when infected with FB29 than with TR1.3. MLV infection of BCEC and KCEC was confirmed by analysis of genomic DNA using PCR assay (data not shown).

TR1.3 MLV selectively induces syncytia formation in BCEC

As an approach to understanding the mechanisms that underlie tissue-specific BCEC cytopathology by TR1.3,

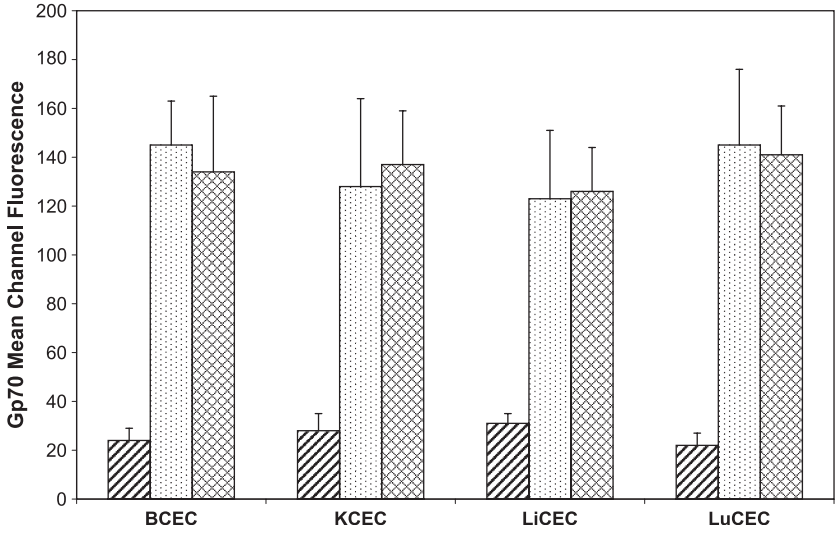

Fig. 3. Expression of gp70 virus glycoprotein in infected CEC cultures. CEC primary cultures from brain (BCEC), kidney (KCEC), liver (LiCEC), and lung (LuCEC) were incubated with either mock virus (striped bar), FB29 (stippled bar), or TR1.3 (hatched bar) and gp70 expression analyzed by immunostaining and flow microfluorimetry. Cells were isolated after 48 h of culture with virus, fixed in paraformaldehyde, and then incubated with goat anti-gp70/rabbit anti-goat FITC reagent. Values are expressed as Gp70 Mean Channel Fluorescence ± the standard error of mean from three separate experiments.

isolated CEC primary cultures were examined for syncytia formation in vitro in response to MLV infection. The capacity of CEC to form syncytia in vitro when infected by either TR1.3 or FB29 was first examined in long-term productively infected primary cultures. The presence of multinucleated giant cell syncytia was observed in TR1.3 infected BCEC cultures by as early as 48 h and persisted for up to 21 days in culture. A representative sample of BCEC syncytia induced by TR1.3 is shown in Fig. 5 (arrow). In contrast to this finding, syncytia were not observed in either KCEC (Fig. 5), or CEC isolated from either liver or lung tissues (data not shown) when incubated with TR1.3. The frequency of syncytia formation in these cultures was 74% in BCEC and <2% in KCEC, LiCEC, and LuCEC. Lastly, in agreement with previous in vivo data, syncytia were never observed during FB29 infection of any CEC type (Chung et al., 1999; Park et al., 1993, 1994a).

Although the formation of syncytia in vivo is generally believed to occur through fusion of infected with uninfected cells, the inherent capacity of cells to form syncytia can also be assessed by susceptibility of two uninfected cells to fuse in the presence of high virus concentrations. Under these conditions, termed fusion-from-without, virus acts as a fusion bridge. To perform this analysis, primary BCEC, KCEC, liver, and lung CEC lines were separately incubated with virus at an MOI of 100–1000 in the presence of 50 μM AZT to block virus replication. Cultures were then observed over 24 h for syncytia formation. As shown in Fig. 6, TR1.3 selectively induced syncytia formation in BCEC, but not KCEC or other CEC (data not shown). The frequency of syncytia formation in these cultures was 43%

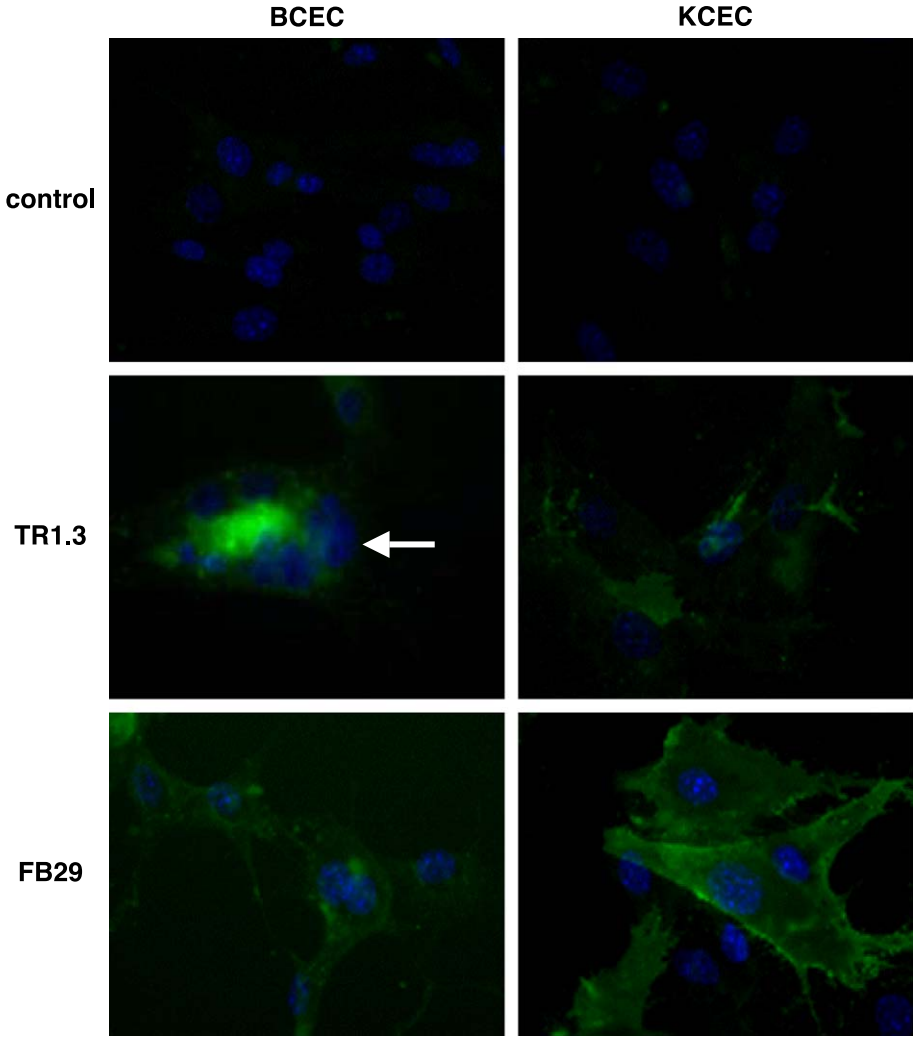

Fig. 5. Cell morphology of MLV-infected BCEC and KCEC in vitro. BCEC and KCEC primary cultures were incubated with either TR1.3 or FB29 (MOI = 1.0) and passed four times in culture. Cells were then fixed (day 18) in paraformaldehyde and stained with goat anti-gp70/rabbit anti-goat FITC reagent (green). Nuclei were stained with DAPI (blue) and visualized under the fluorescence microscope (magnification, 200×). The presence of a multinucleated giant cell is indicated by the arrow. The data are representative of three independent experiments.

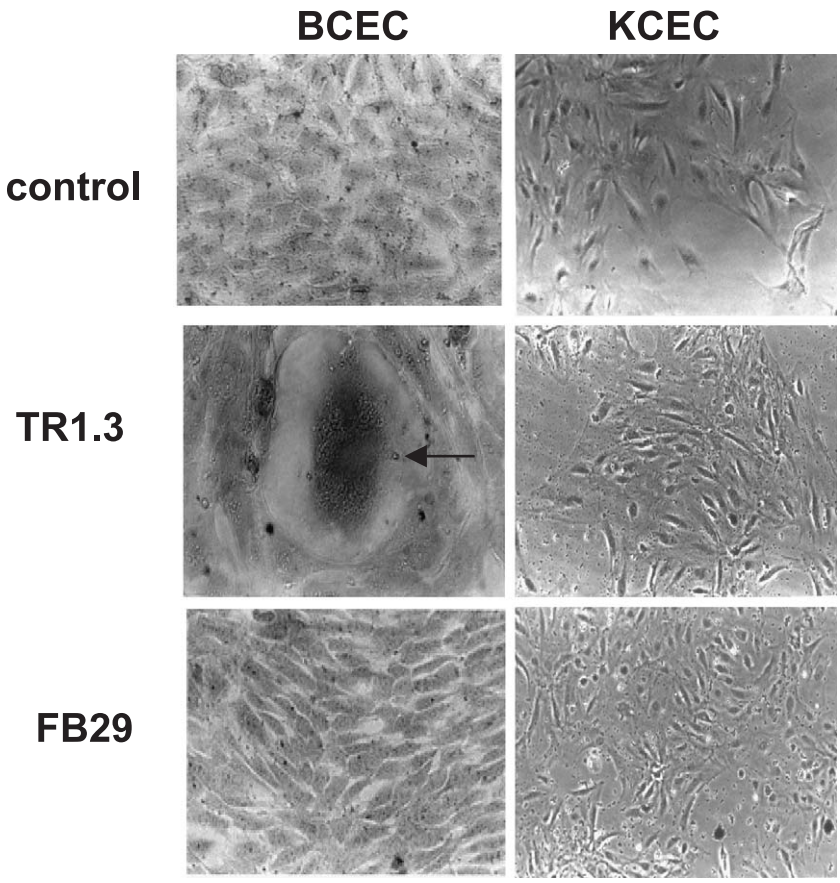

Fig. 6. Fusion-from-without in BCEC primary cultures incubated with TR1.3 MLV. Subconfluent monolayers of primary BCEC and KCEC lines were incubated with concentrated, purified virus or control in the presence of 50 μM AZT for 18 h. Cells were stained with methylene blue and examined microscopically: magnification BCEC, 200×; KCEC, 50×. Data shown are representative of three independent experiments. The presence of a multinucleated giant cell is indicated by the arrow.

in BCEC and <2% in KCEC, LiCEC, and LuCEC. Syncytia were not observed in any CEC type when incubated with FB29 MLV.

Discussion

TR1.3 infection of BALB/c mice results in syncytia formation of infected BCEC but not CEC of other organs (Park et al., 1993). In contrast, exposure of mice to the closely related virus FB29 does not induce syncytia formation in any CEC type despite widespread CEC infection. These observations suggest that syncytia formation is regulated by properties inherent to both virus and host cell type. The fusion of virus with cell membranes is a multistep process (White, 1992). Viral envelope proteins first interact with receptors on the host cell. In the instance of MLV, this is believed to occur solely through interaction with the ecotropic receptor MCAT-1, although accessory components have been suggested (Kizhatil and Albritton, 1997; Siess et al., 1996). The fusion process is initiated by insertion of the hydrophobic transmembrane domain of MLV envelope within the cellular plasma membrane (Fass et al., 1997). Membrane mixing occurs when a sufficient density of multimeric receptor–envelope complexes are brought into close proximity. The events that govern virus-mediated cell–cell fusion are less well understood, but are assumed to follow a similar process.

To examine these cellular properties, we established an in vitro model of MLV infection in primary CEC. We first established primary CEC cultures that were 95% pure, as determined by Factor VIII expression, and that showed the same rate of cellular proliferation. This is critical in that MLV is acutely dependent on cell replication for virus integration. We then examined infection of CEC types using pathogenic TR1.3 and nonpathogenic FB29 MLV isolates. Kidney, liver, and lung tissues were chosen for study as these organs were previously demonstrated to show TR1.3 infection in vivo (Park et al., 1993). All endothelial cells were productively infected to an equal degree by TR1.3 and FB29 as determined by viral integration, infectious virus production, and viral envelope staining. Lastly, direct analysis of syncytia formation in purified in vitro CEC cultures was conducted on both productively infected cells and on uninfected cells using the fusion-from-without assay. In both instances, syncytia were observed upon infection of

BCEC with TR1.3. In marked contrast, syncytia were neither detected in TR1.3 of other CEC types nor were observed in any CEC type upon infection with the nonpathogenic MLV FB29. Together these results demonstrate that the in vitro system defined here recapitulates the virus and cell selectivity seen during in vivo infection with TR1.3 (Park et al., 1993, 1994a). The results reinforce our published results that TR1.3 is inherently more fusigenic than FB29, and demonstrate that the differential fusion sensitivity of BCEC to TR1.3 is linked to unique attributes of BCEC.

There are several possible means whereby BCEC may display enhanced fusion capacity. The level of virus Env and receptor protein expressed on the plasma membrane play an important role in regulating the extent of syncytia formation. In previous studies, we demonstrated that there was a minimal MCAT-1 receptor threshold level for initiation of fusion, and that this threshold is lower for TR1.3 than FB29 envelope (Chung et al., 1999). We also showed that the nonpathogenic MLV FB29 mediated cell fusion at a level equal to that of TR1.3 when envelope and receptor were overexpressed on target and effector cells, respectively (Chung et al., 1999). Our analysis here showed that both the production of infectious TR1.3 and FB29 particles, and gp70 Env protein expression were similar in infected CEC lines.

Whether alterations in either receptor levels, accessibility, or surface distribution contribute to enhanced BCEC syncytia formation remain interesting possibilities. For example, if the absolute amount of MCAT-1 expression on BCEC is augmented relative to other CEC types, this may permit syncytia formation by TR1.3 but not FB29, as TR1.3 requires a lower receptor threshold to initiate fusion. Metabolic modifications of the MCAT-1 receptor, such as glycosylation, can also have profound effects on envelope binding and fusion (Eiden et al., 1994; Miller and Miller, 1992; Wilson and Eiden, 1991), and glycosylation patterns of MCAT-1 may differ among CEC in different organs. Lastly, as BCEC form tight junctions and have unique transport properties, the accessibility and distribution of receptor on the cell surface may vary among CEC types (Rubin and Staddon, 1999; Woodard et al., 1994). Irrespective of receptor levels or posttranslational modification, BCEC may also possess unique cellular properties that render them more acutely sensitive to cell fusion. For example, BCEC may exhibit a distinctive cytoskeletal organization or membrane composition that promotes either cell adhesion or fusion (Hildreth and Hampton, 1997; Wilson et al., 1992). Alternatively, KCEC and other CEC types may possess a cellular factor that limits membrane fusion (Siess et al., 1996). We are currently examining each of these possibilities in CEC isolated from different organs.

In conclusion, this study highlights the establishment of an in vitro model of TR1.3-induced syncytia formation in BCEC. With this system, we demonstrated that the selective in vivo formation of BCEC syncytia during TR1.3 MLV infection is a unique property of BCEC as contrasted to CEC resident in other tissues. Further insights into the role of BCEC-specific cellular factors that regulate syncytia formation are an essential element in understanding virus-induced neuropathology and neurological disease.

Materials and methods

Primary endothelial cell cultures

Primary cultures of CEC were prepared from the brain (BCEC), kidney (KCEC), liver (LiCEC), and lung (LuCEC) microvessels of 28-day-old BALB/c mice using a modification of a previously defined protocol (Hoffman et al., 1992). All experiments were performed in accordance with guidelines using IACUC approved protocols. Briefly, CEC were grown in minimum essential medium (MEM) with D-valine (Life Technologies, Inc., Gaithersburg, MD) supplemented with 20% fetal calf-serum, L-glutamine (2 mM), penicillin (50 u/ml), streptomycin (50 μg/ml), 1/100 volume of MEM nonessential amino acids solution (Life Technologies, Inc.), heparin (16 u/ml), and endothelial cell mitogen (20 μg/ml) (Biomedical Technologies Inc., Massachusetts). Aliquots of each primary culture were passed 4–7 times and examined for purity by expression of Factor VIII using indirect immunofluorescence. Greater than 95% of CEC cells in culture expressed Factor VIII.

Endothelial cell growth was measured by thymidine incorporation, as previously described (Park et al., 1994c). In brief, 1×10^2 CEC cells were plated in 96-well plates and pulsed for 6 h with 1 μCi per well [^{3}H]-thymidine (Dupont NEN, Waltham, MA) after 24, 48, or 72 h in culture. Cells were harvested with a PHD harvester (Cambridge Technology Inc., Cambridge, MA) and incorporated radiolabel measured in a liquid scintillation counter.

Virus isolation and infection

The production of FB29, TR1.3, and W102G infectious virus was previously described (Park et al., 1994c; Sitbon et al., 1986). In brief, FB29, TR1.3, and W102G are long terminal repeat-permuted viral DNAs that were cloned into the modified vector pUC19B. Infectious virus was produced by gel isolation from pUC19B with *Hin*dIII digestion. Virus was then circularized with T4 DNA ligase and transfected into the feral mouse embryoblast cell line SC-1 (American Type Culture Collection, Rockville, MD), using a modified calcium phosphate transfection procedure as described previously (Wigler et al., 1979). SC-1 cells were grown in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. After 1–2 weeks in culture, supernatants were harvested and assayed for the presence of reverse transcriptase (RT) activity (Goff et al., 1981). Viral supernatants were aliquoted and stored at −80 °C. Viruses were purified by ultracentrifugation on a 20% sucrose cushion at 19 500 rpm

for 2 h (Burns et al., 1993). Viral pellets were resuspended in DMEM in 10% original volume. Virus titers were determined by a modified XC cell plaque assay as described by Rowe et al. (1970).

Endothelial cells were seeded at 1×10^6 cells per TC-75 flask in 10 ml of media. To initiate virus infection, the culture media was reduced to 1 ml, supplemented with 4 μg/ml polybrene (Sigma Co., St. Louis, MO) for 1 h, and virus added at an MOI of 1.0 for 1 h at 37 °C. Nine milliliters of media containing polybrene was then added to each flask and incubation continued for 24 h. After this time point, CEC were washed extensively to remove residual virus and fresh media added. Cultures were subsequently re-fed with fresh media on days 3 and 6 before passage. Infected and uninfected cultures were harvested for analysis at time points indicated in the Results.

Syncytia formation and fusion assay

The formation of cell syncytia in virus infected cultures was detected by microscopic examination of cell morphology after 1–21 days. Cells were fixed in methanol (−20 °C for 3 min) and stained with 0.5% methylene blue in PBS. Quantification of syncytia was performed by counting cells in 10 random microscopic fields under 100–400× magnification. Fusion-from-without assays were conducted by plating CEC in 24 well plates at $1–2 \times 10^5$ cells per well. After overnight incubation, cells were treated with 50 μM AZT in media for 12 h. Equivalent amounts of purified viruses (MOI = 100–1000) were added in the presence of 8 μg/ml polybrene and allowed to incubate overnight. Syncytia formation was determined as described above. The frequency of syncytia formation in cultures was calculated by dividing the total number of syncytia by the number of fields counted. A total of 200 fields were counted twice for each cell line.

Immunohistochemistry

Immunohistochemical detection of virus and cellular antigens was performed on BCEC and KCEC plated ($0.5–1.0 \times 10^3$ cells/well) on poly-L-lysine coated coverslips and fixed in 4% paraformaldehyde for 20 min at room temperature. Intracellular protein expression in CEC was performed after fixation in 80% acetone at −20 °C for 5 min. Indirect immunofluorescence was performed after blocking of coverslips with 5% normal donkey serum (Jackson ImmunoResearch Labs, West Grove, PA) for 30 min at room temperature. Coverslips were then labeled with a 0.1% solution of rabbit antihuman Factor VIII (DAKO Corporation, Carpenteria, CA) and a 0.05% solution of goat anti-Rauscher gp70 (Quality Biotech Inc., Camden, NJ) primary antibodies overnight. Coverslips were next rinsed with PBS and then incubated with a 1% solution of either donkey anti-rabbit FITC or TRITC (Jackson ImmunoResearch Laboratories, Inc., Bar Harbor, ME), or rabbit anti-goat TRITC (Sigma) secondary antibodies for 1 h at room temperature. The coverslips were next rinsed in PBS, mounted, and sealed before analysis by fluorescence microscopy.

Acknowledgments

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